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**13. Abstract** ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Dendritic cells (DC) are an integral part of the immune system's response to cancer. A number of clinical trials have been initiated to use these cells in the treatment of melanoma, prostate cancer, and lymphoma. In preparation for treating breast cancer patients with DC, this proposal examines a fundamental issue that needs to be resolved before proceeding with this exciting new therapy. We hypothesized that the processing and presentation of multiple tumor antigen epitopes by DC is a more efficient and effective way of stimulating T cell responses than current HLA-restricted peptide-based methods. The goal of this proposal is to develop practical methods by which immune cells from patients with breast cancer can be used to promote effective anti-tumor responses. In the past year, we have continued evaluation of patient DC, developed methodology for cross-presentation of multiple tumor antigens by apoptotic tumor cell-loaded DC, completed development of assays to assess anti-tumor reactivity, and initiated experiments to compare the various antigen-arming methods. This progress is consistent with the proposal's Statement of Work, and leaves us well-positioned to complete the next goals of the proposal.

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## INTRODUCTION

Dendritic cells (DC) are an integral part of the immune systems' response to cancer. When loaded with tumor antigens, DC have great value as immunotherapeutic agents. However, the method for arming the DC with antigens which results in the most effective immune response has yet to be defined. We theorized that the broadest possible mix of tumor antigens might provide the best material for stimulating an effective immune response. We hypothesized that the processing and presentation of multiple tumor antigen epitopes by DC would be the most efficient and effective way of stimulating T cell responses. The goal of this proposal is to develop practical methods by which immune cells from patients with breast cancer can be used to promote effective anti-tumor responses. In this study we will compare multiple methods of arming DC with tumor antigens including: 1) purified immunodominant peptides which are specific for a single antigen and a single Class I MHC molecule, 2) transduced cDNA encoding for a single tumor antigen which will allow the recipient DC to intrinsically process and present all possible antigenic peptides (immunodominant and sub-dominant) within the context of all available MHC molecules, and 3) extracts from autologous or allogeneic whole tumor cells which will provide a broad mix of tumor antigens (both defined and undefined antigens) for processing and presentation. The information obtained from this study will further our understanding of the interactions between DC and T cells which lead to the generation of tumor-antigen-specific responses. This understanding will be valuable in the development of immunotherapeutic treatments for breast cancer.

## ANNUAL SUMMARY

RESEARCH ACCOMPLISHMENTS:

For the year 7-1-00 through 6-30-11 (proposal months 25-36), we are pleased to report progress on Tasks 1-4 in the proposal Statement of Work. This progress is composed of advances in four main research areas: evaluation of patient DC, development of the apoptotic tumor methodology, development of assays to assess anti-tumor reactivity, and initiation of experiments to compare the antigen-arming methods.

Task 1. To identify HLA-A2$^+$ breast cancer patients whose tumors do and do not overexpress Her-2/neu.

*Evaluation of Patient Dendritic Cells*
Research by ourselves and others has demonstrated that the tumor environment has an immunosuppressive effect on DC. Many of these studies have been done on pathology specimens in a retrospective fashion. Studies determining the immune status of patients' DC in a prospective manner are needed. In the past year, we have continued the characterization of the phenotype and functional activity of the breast cancer patients' DC. As a corollary to these studies, we are investigating ways to enhance the phenotype and function of the patient's DC by either adding stimulatory cytokines or blocking inhibitory cytokines during the maturation process. These approaches may be necessary to optimize the ability of patient's DC to function as a vaccine in future clinical studies. We had found previously that the addition of anti-IL-10 to the cultures during DC generation improved the development of normal subject's DC, perhaps by blocking endogenous IL-10 produced by the monocytes. IL-10 may also be present in the local tumor environment. Interestingly, when we added anti-IL-10 to one patient's DC culture, the antibody had the opposite effect as on the normal subject's DC and actually impaired the phenotypic maturation of the DC. Although these results are very preliminary, they highlight the importance of studying patient's DC, since they may behave differently than those of healthy subjects.

Task 2. To obtain peripheral blood and tumor specimens from these patients and use them to generate DC, isolate T cells and produce tumor cell lysates.

In the past year, the development of the methodology required for the accomplishment of Task 2 has been completed. This methodology utilizes the latest techniques in antigen-loading, and its development leaves us favorably positioned to complete Tasks 3 and 4 in the next year.

*Development of apoptotic tumor-loading methodology*
As described in earlier annual summaries, we have proposed using apoptotic tumor cells as an alternative to tumor lysate as a source of multiple tumor antigens. This increases the number of patients with can be enrolled in the study (and potentially enrolled in future treatment protocols) because it is not necessary to have access to tumor specimens from the patients. We can use apoptotic allogeneic tumor cells because an HLA-match is not required. In the past year, we have developed a UV-based method for inducing apoptosis in breast cancer cells. Within 24 hours of the induction of apoptosis, >90% of the tumor cells are apoptotic as assessed by annexin/propidium iodide staining. The next step in this procedure was to determine the optimal conditions for the uptake of the apoptotic cell bodies by DC. We stained for intracellular Her-2 in DC which had been

co-cultured with apoptotic bodies from the Her-2 overexpressing breast cancer cell line SKBr3. Ratios of 1:1, 1:0.5 and 1:0.1 DC:tumor were used. As expected, the higher ratio produced the greatest percentage of Her-2-containing DC, with a minimal amount of unincorporated tumor. In an additional test, DC and tumor were labeled with the membrane stains PKH67 (green) and PKH26 (red), respectively. Apoptosis was induced in the labeled tumor cells, and after 24 hours they were mixed with DC at a 1:1 DC:tumor cell ratio. As shown in Figure 1, after 2 hours of co-culture >50% of the DC had taken up apoptotic cell bodies. Greater than 90% of the DC which were co-cultured for 22 hours incorporated tumor.

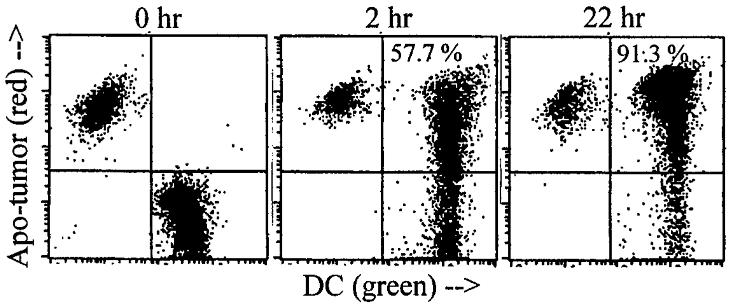


**Figure 1. DC take up apoptotic tumor cells in a time-dependent manner.** DC (labeled with PKH67) and apoptotic cell bodies (labeled with PKH26) were admixed at a 1:1 DC:tumor cell ratio. Cells were collected at the various time points, fixed with paraformaldhyde and analyzed by FACS analysis. DC in the upper right quadrant contain captured tumor.

Task 3. <u>To determine the frequency of Her-2-specific T cells generated using the different antigen-arming methods with a modified limiting dilution procedure</u>.

It is our hypothesis that the processing and presentation of multiple tumor antigen epitopes by DC is the most efficient and effective way of stimulating T cell responses. With this multiple tumor antigen approach, we expect an increased frequency of responding T cells and a broadening of the response to the tumor. This broader response may result in increased effectiveness *in vivo* due to greater tumor killing, and the prevention of the selection of resistant variants. Task 3 and 4 involve evaluating the different antigen-loading methods for their ability to stimulate tumor-specific T cell responses. In the past year, we have finished developing the assays to assess anti-tumor reactivity, and have initiated experiments to compare the antigen-arming methods.

*Assay Development*

In the previous grant years, we had developed assays to assess antigen-specific cytotoxicity (Alamar Blue) as well as ELISpot assays for cytokine-producing cell frequency. The final assay which needed to be developed was intracellular cytokine staining (ICS). This is a sensitive, fluorescence-based assay which can detect the cytokines produced by a single cell. Because of the variety of fluorescently-labeled anti-cytokine antibodies now available, a single cell can be assessed for its ability to produce multiple cytokines in a single assay. In the past year, we have optimized the conditions for eliciting cytokine production, as well as the processing and staining procedures. An example of the results of this assay is shown in Figure 2.

*Evaluation of Antigen-arming Methods*

In the past year, we have begun to evaluate the ability of the various antigen-arming methods to stimulate tumor antigen-specific T cell responses. The initial experiments were designed to determine optimal conditions for producing maximal antigen-specific reactivity, with minimal non-specific reactivity. A number of DC:T cell ratios were evaluated, as well as the use of stimulatory cytokines. We determined that the optimal ratio of DC:T cells was 1:20, and that the addition of 2 ng/ml IL-7, and 10 IU/ml IL-2 aided T cell development without inducing non-specific responses. The second set of experiments evaluated the number of weekly restimulations necessary to achieve antigen-specific T cell responses. We found that the optimal number of restimulations varied with the antigen-arming technique. With apoptotic tumor-loaded DC, the T cells showed measurable reactivity at 10-14 days of culture (2 stimulations), while T cells stimulated with E75 Her-2 peptide required at least 21-28 days of culture (3-4 stimulations). At these later time points, the T cells which had been stimulated with apoptotic tumor-pulsed DC were undergoing activation-induced cell death. We have decided to assess T cell responsiveness on a weekly basis, in order to ensure that the various antigen-arming techniques are evaluated appropriately. Figure 2 below shows the anti-Her-2 reactivity induced after 2 stimulations (14 days of culture) with apoptotic SKBr3-loaded DC. T cells which had been stimulated 2 times with E75-loaded DC showed only minimal reactivity at this time point. The T cells were evaluated for IFN-γ and IL-10 production by intracellular cytokine staining following a 5 hour stimulation with unloaded DC, apoptotic MCF7/neo-loaded DC, or apoptotic MCF-7/Her-2 loaded DC in the presence of anti-CD28, 1 ng/ml IL-12, and brefeldin A.

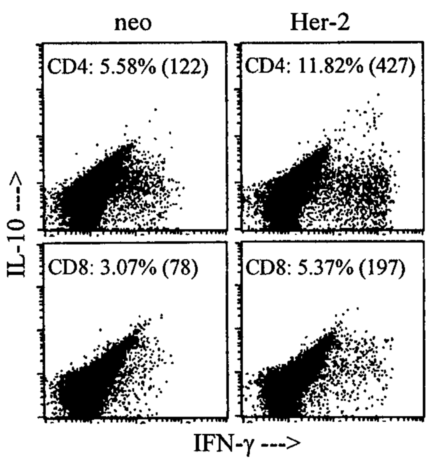


**Figure 2. T cells show enhanced responsiveness to Her-2 as assessed by IFN-γ production.** T cells were restimulated on a weekly basis with DC pulsed with apoptotic SKBr3 (Her-2+) cells. Intracellular IFN-γ production was stimulated by DC pulsed with either apoptotic MCF-7/neo or apoptotic MCF-7/Her-2 in a 5 hour assay. The percent positive cells are listed for both CD4 and CD8 T cell populations. Numbers in parentheses denote the fluorescence intensity of the IFN-γ positive cells. T cells which had been repeatedly stimulated with DC pulsed with the E75 peptide (instead of apo-SKBr3) showed minimal (< 1.5%) responsiveness to Her-2 in the 5 hour assay.

Preliminary results suggest that apoptotic tumor-loaded DC induce a larger number of specific T cells at an earlier time point than E75-peptide-loaded DC. Interestingly, $CD8^+$ T cell responses are improved when the $CD8^+$ cells are stimulated in the presence of $CD4^+$ cells, as opposed to purified before stimulation.

| | % CD8 positive for IFN-γ | Intensity of IFN-γ expression (LFI) |
|---|---|---|
| CD8 alone | 0.39 | 88 |
| CD8 with CD4 | 3.19 | 286 |

**Table 1. Proportion of $CD8^+$ cells which respond to Her-2 antigen with IFN-γ production increased when $CD8^+$ cells were cultured in the presence of $CD4^+$ cells.**

As shown in Table 1, $CD8^+$ cells which were stimulated in the presence of $CD4^+$ cells had a 10 times higher percentage of IFN-γ-producing cells. In addition, these cells produced more IFN-γ on a cell-by-cell basis as assessed by intensity of antibody staining. These results underscore the importance of helper T cells in the development of anti-tumor responses. In future experiments, we will continue to use both CD8 purified and CD3 cells as responding cell populations in order to further examine this effect.

Task 4. To characterize the response obtained by stimulation of T cells by DC.

Task 4 involves characterizing the T cell response to Her-2 to determine the proportion of cells which respond to 1) Her-2-E75 peptide, 2) other epitopes of Her-2, or 3) other undefined antigens expressed by the patient's tumors. In the past year, we have updated the methodology to assess these responses, and in conjunction with Task 3, have begun to evaluate the T cell responses induced by the various antigen-arming methods.

*Tetramer Staining*
The original protocol proposed evaluating the contribution of anti-E75 peptide response to the total anti-tumor response by attempting to block specific T cell-tumor reactions with the addition of excess peptide. Since the proposal was written, advances have been made in the technology available to assess peptide-specific responses. Chief among these is MHC-tetramer staining-- an increase in tetramer-positive cells following treatment has been associated with enhanced clinical responses. HLA-A2-tetramers specific for a variety of peptide antigens are now available, including the E75 peptide of Her-2/p185. We have purchased this tetramer (labeled with phycoerythrin), and are using it to characterize the anti-tumor response generated by the various antigen-armed DC in experiments that are currently underway.

*Evaluation of multiple epitope responses*
While evaluation of the response to the E75 peptide has been simplified by the advent of labeled MHC tetramers, the evaluation of the response to Her-2 and undefined antigen is more complicated. In our study, T cells are stimulated with DC pulsed with E75 peptide, apoptotic tumor (either SKBr3 or autologous), or transduced with AdV-Her-2. Both SKBr3 and the patient's tumor express HER-2, as well as a variety of other defined and undefined antigens. To assess what proportion of the response is directed against Her-2, we have utilized two cells lines developed from the breast cancer cell line MCF-7. MCF-7/Her-2 is an HLA-A2$^{+}$ cell line which has been retrovirally transduced with Her-2. MCF-7/neo has been retrovirally transduced with a similar vector lacking the HEr-2 transgene. Following *in vitro* stimulations with DC pulsed with E75, apoptotic tumor, or transduced with AdV-Her-2, the T cells are collected and evaluated for their ability to respond to DC which are unpulsed, pulsed with apoptotic MCF-7/Her-2, or pulsed with apoptotic MCF-7/neo. The specific response to Her-2 can be assessed by the difference between the neo and Her-2 responses (see Figure 2). The responses to shared antigens other than Her-2 can be assessed by the difference between unpulsed and MCF-7/neo-pulsed DC.

TRAINING ACCOMPLISHMENTS:

In the past year, I became a member of the UCLA Jonsson Comprehensive Cancer Center. Privileges of membership include access to financial support and office and research space controlled by the Cancer Center, as well as priority access to shared resources and core services. Later this summer, I will be moving my laboratory into newly remodeled laboratory space.

I am planning on submitting two grant applications in Fall 2001, an NIH R01 grant application and an American Cancer Society Research Scholar Grant application. These studies will be based on the results generated by this proposal.

In summary, the progress in the past year is consistent with the proposal Statement of Work, and leaves us well-positioned to complete the next goals of the proposal.

## KEY RESEARCH ACCOMPLISHMENTS

- Renewed IRB approval for recruitment flyers and informed consent forms
- Continued assessment of phenotypic and functional activity of patient's circulating and monocyte-derived DC
- Developed methods to produce apoptotic tumor cells using UV irradiation.
- Optimized procedure for loading of DC with apoptotic tumor cells
- Obtained HLA-A2-MHC-tetramer for the Her-2 E75 peptide.
- Initiated evaluation of antigen-arming methods for their ability to generate antigen-specific T cells.

**REPORTABLE OUTCOMES**

1. Membership in UCLA Jonsson Comprehensive Cancer Center approved.

2. Attended Keystone Symposium: "Dendritic Cells: Interfaces with Immunobiology and Medicine", Taos, NM, March 12-18, 2001.

3. Moving to newly remodeled laboratory space in late summer 2001.

4. Planning NIH R01 grant application for 10-01-01deadline.

5. Planning American Cancer Society Research Scholar Grant application for 10-15-01 deadline.